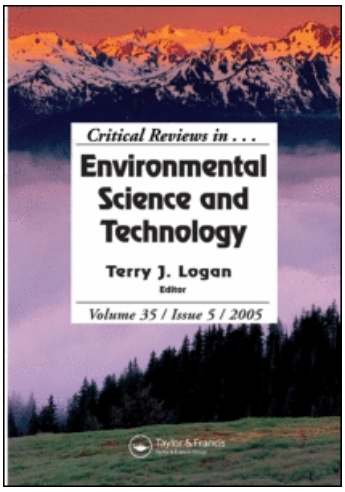

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Environmental Dissemination of Foodborne *Salmonella* in Preharvest Poultry Production: Reservoirs, Critical Factors, and Research Strategies

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The impact of potential pathogenic foodborne Salmonella *spp. in poultry production environments is of paramount importance, considering its implications for human health. Most of what is known about this organism under these environmental conditions is based on indirect evidence. The overall focus of this review is on the biology of potentially pathogenic foodborne* Salmonella *spp. in poultry environments. This is not just because of the implications regarding pathogenic* Salmonella *spp. for poultry production and food safety but because* Salmonella *spp. behavior may serve as a model for understanding general bacterial pathogen persistence in animal agricultural environments. This will help meet a long-term need to develop a comprehensive ecological picture of the contamination potential, growth, survival, and genetic stability of pathogens in poultry and other animal production environments. This will in turn lead to a better understanding of the environmental and health impacts of foodborne* Salmonella *spp. dissemination in animal production environments.*

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I. INTRODUCTION

As municipal populations continue to increase and add to urban sprawl, agricultural lands are lost. At the same time, the livestock industry continues to increase the number of animals per unit, resulting larger and larger confined animal feeding operations. The interface resulting from these two trends can have disastrous consequences, as demonstrated by the extensively documented series of environmental catastrophes associated with intensive animal production waste-handling facility failures.[1] In 1995, several swine-waste lagoon spills took place in North Carolina after heavy rains in river basins followed by the flooding associated with hurricanes in succeeding years. The nutrient-rich loads caused by overflowing lagoon waste material lead not only to fish kills by algal blooms but substantial damage to the aquatic ecosystems due to overloads of nutrients from the lagoon.[1–4] In addition, the microbial load originating from animal fecal material contained pathogenic bacteria, protozoa, and viruses that had the capability of infecting humans.[1,5] Although this scenario represents an out of control disaster magnified by the sudden impact of several severe hurricanes in rapid succession, high concentrations of animals in close proximity can still have less obvious but wide-ranging consequences on environmental quality. It is clear that failure to recognize environmental impacts can lead to substantial environmental and human health repercussions for the animal industry. With the poultry industry having developed into an intensive animal production for the longest period of time, producers have perhaps been confronted with environmental consequences sooner than for most phases of animal agriculture.

In addition to the chemical pollutant problems commonly identified with animal wastes, such as poultry manure and litter/manure, are the wide varieties of microorganisms, originally harbored in the intestinal tracts, which continue to survive in the waste materials. Many of the bacteria that can cause diseases in humans can be found among these organisms. Poultry can be one of the most important animal reservoirs of asymptomatic *Salmonella* spp. in the human food chain.[6] The overall focus of this review is on the study of potentially pathogenic *Salmonella* spp. in poultry production environments. This not just because of the implications regarding pathogenic *Salmonella* spp. for poultry production and food safety but because *Salmonella* spp. behavior may serve as a model for understanding general bacterial pathogen persistence in animal agricultural environments. To present this picture, several objectives are developed in this review. The first objective is to provide a brief overview of public health problems associated with poultry production

environments particularly as a potential reservoir for foodborne pathogens. The pathogen of primary interest here is *Salmonella*. This is because of the importance of this organism in foodborne disease outbreaks and the consistent association of this organism with all aspects of poultry production and its subsequent persistence in the environment. Since most of the research is on foodborne aspects of salmonellae and food safety-related characterization, a portion of this review is used to discuss the methodology available to isolate, quantify, and characterize this organism in poultry environments. In addition, the survival characteristics and genetics of salmonellae under these environmental conditions have largely been ignored. However, several research techniques are now available to assess the physiology of salmonellae in these settings, and a more complete picture of *Salmonella* spp. is now feasible. Therefore, the potential importance of survival response is examined and we include some discussion on methods that are recently available for tracking and studying *Salmonella* under environmental conditions encountered during poultry production. Finally, future concerns and directions are examined.

II. *SALMONELLA AND HUMAN SALMONELLOSIS*

Salmonella are facultative, gram-negative, and nonsporing rods in the family Enterobacteriaceae.[7] *Salmonella* serovars can be divided into three groups based on the epidemiological purposes.[8] Group I correspond to the infection of humans only. These include *S. typhi*, *S. paratyphi* A, *S. paratyphi* C, and the agents of typhoid and paratyphoid fevers; group I causes the most severe of all human diseases caused by salmonellae. Group II correspond to the host-adapted serovars, some of which are human pathogens and may be contracted from foods. These include *S. gallinarum* (poultry), *S. dublin* (cattle), *S. abortus-equi* (horses), *S. abortus-ovis* (sheep), and *S. choleraesuis* (swine). Group III correspond to unadapted serovars, those with no host preference. These include most foodborne serovars and pathogens for humans and other animals. *Salmonella* species and serovars can cause several diseases such as gastroenteritis and systemic disease in different hosts.

Salmonella enterica serovar Enteritidis and Typhimurium (referred to as *S. enteritidis* and *S. typhimurium* through the remainder of this review) are of public health significance due to their more complex epidemiology involving extensive fecal excretion with associated environmental contamination and the existence of many different infections.[9] *Salmonella enteritidis* and *typhimurium* are reported to be the most common pathogens of human salmonellosis.[10] Salmonellosis is one of the primary foodborne diseases, with an estimated 800,000 to 4 million human infections reported each year in the United States alone,[11,12] and represents an annual loss of approximately

$4 billion from the U.S. economy, including reduced productivity and medical treatment costs.[13] Approximately 60% of U.S. human salmonellosis in 1995 was caused by *S. enteritidis* (24.7%) and S. *typhimurium* (23. 5%).[14]

In the last 10 years, the infection of greatest concern in the United States has been caused by *S. enteritidis*.[12,15] In 1993, a large outbreak in Florida was caused by *S. enteritidis* in ice cream produced from milk that was transported in tanker trucks that had previously hauled unpasteurized liquid eggs.[16] Therefore, *S. enteritidis* and *S. typhimurium* continue to be the leading causes of foodborne salmonellosis in humans in the United States. According to Tauxe,[17] four current trends associated with foodborne *Salmonella* disease are likely to continue to present important public health challenges: (1) increasing antimicrobial resistance, (2) the intersection of salmonellosis and the acquired immunodeficiency syndrome (AIDS) epidemic, (3) egg-associated *Salmonella* infections, and (4) large and dispersed outbreaks.

Poultry is considered one of the most important sources of salmonellae[18–21] and constitutes an important animal reservoir of asymptomatic *Salmonella* excreters in the human food chain.[6] Because the supply of eggs or chicks from infected breeder flocks can lead to a significant increase in the degree of infection of progeny, salmonellae infection at early stages of production is not only critical[22] but can result in an ever-cascading influence on poultry production at later stages. Since salmonellae are a public health hazard, it is essential that efforts be directed toward monitoring potential environmental sites for contamination throughout poultry production.

III. ROUTES OF DISSEMINATION OF *SALMONELLA* WITHIN THE POULTRY PRODUCTION CYCLE

A. Vertical Versus Horizontal Contamination

Vertical contamination is transovarian transmission of microorganisms from hens to progeny or table eggs.[23] This transmission rapidly increases the incidence of disease in poultry flocks and permits cross-contamination due to contamination of the egg passage via the chicken.[23] When this occurs, there is the potential for an immediate amplification of the number of birds to be *Salmonella* positive and consequently there are greater opportunities for horizontal contamination and spread of the organism. Horizontal contamination is the transmission of microorganisms via direct contact of uninfected birds with infected birds, or indirectly by contaminated drinking water, feed, or airborne horizontal transmission of microorganism from the infected bird to uninfected neighboring birds.[24–26] High bird density in commercial poultry houses may lead to increases in *Salmonella* spread by these indirect poultry-house environmental routes. Specific contamination routes and potential environmental contact points are discussed in the following sections.

B. Eggborne Sources

An important source of human *Salmonella* infection is contaminated eggs or egg products[19,27]; this makes it a unique threat to food safety. The on-farm environment of the chicken is a rich source of a number of *Salmonella* serotypes,[28,29]and *S. enteritidis* is a primary pathogen that causes frequent human illness associated with egg contamination. Although laying hens may harbor *S. enteritidis*, they show no clinical signs of infection, and the eggs they produce appear normal.[30] Egg contamination by *S. enteritidis* has been a continuing international public health problem for more than a decade.[12] Foodborne outbreaks of *S. enteritidis* infection in the United States for which vehicle could be identified were most often associated with Grade-A shell eggs.[19,27,31]

Egg contamination results from penetration through the eggshell by *S. enteritidis* contained in feces after the egg is laid, horizontal transmission,[32–35] or by direct contamination of reproductive organs with *S. enteritidis* before the egg is laid, vertical transmission.[36,37] *Salmonella gallinarum* and *S. pullorum* are strict host-adapted serovars that can infect the ovary and cause transovarian transmission into the egg.[38] *Salmonella heidelberg*, *S. kentucky*, *S. saintpaul*, *S. thompson*, *S. typhimurium*, and *S. hadar* are non-host-adapted serovars that have been isolated from the reproductive organs of hens and from egg contents.[38–41]

Salmonella enteritidis may be carried in the large intestines of adult laying hens and shed in their feces, which may lead to contamination of the egg shell surface by *S. enteritidis*.[42] In addition, *S. entertidis* oral inoculation to adult laying hens can infect the reproductive tracts following localization and colonization of the large intestine, that is, by transovarian transmission, although there are no noticeable clinical signs in the hens.[36,37] Ovarian infections of *S. entertidis* cause both the laying of contaminated eggs and the hatching of infected chicks from the contaminated hatching eggs. These infected chicks grow up to become pullets and subsequently lay contaminated eggs.[43,44]

C. Feed Sources

Feed is an important source of *Salmonella* contamination in poultry.[45–52] Feed serves as a source of horizontal transmission once it has been contaminated by feathers, feces, or airborne *Salmonella*.[53] Veldman et al.[52] reported that *Salmonealla* contamination rates for mash layer-breeder feeds (21.4%) were higher than those of pellet layer-breeder feeds (1.4%). Jones et al.[21] also reported that *Salmonella* contamination rates for mash type feeds (35%) were higher than those of pellet type feeds (6.3%) at the feed mills. Therefore, pelleting poultry feed is a potential management option for reducing the incidence of contamination with *Salmonella*.[52] However, layer-breeder feeds

are usually not pelleted; this prevents dirty egg shells and maintains a low level of feed intake by birds.[52,54] Mash layer-breeder feeds with acid can reduce the vertical transmission of *Salmonella*.[54,55] Fish meal, meat and bone meal, tapioca, and maize grits used as feed components for layer-breeder feeds influence the incidence of *Salmonealla* contamination with 31, 4, 2, and 27%, respectively, for feeds formulated with each ingredient.[52]

D. Other Sources in the Laying-House Environment

In both broiler houses where birds are grown for meat production and egg-laying facilities where eggs are produced, there are several environmental sources that could be commonly found in both facilities. The discussion here is limited to laying houses that have been characterized extensively in pursuit of identification and characterization of environmental sources for *S. enteritidis* during egg production. Numerous potential sources of *Salmonella* exist in the laying house environment.[56,57] Jones et al.[57] reported that *Salmonella* was isolated from egg belts (72.7%), egg collectors (63.6%), ventilation fans (100%), and flush water (100%) in the commercial laying-house environment. Murase et al.[58] suggest that *Salmonella* spp can be spread via egg belts from one house to others. Garber et al.[30] reported that overall 7.1% of layer houses and 3.7% of mice were culture positive for *S. entertitidis* from a total of 200 layer houses and 129 house mice, respectively, in 15 states throughout the United States. Mice may both amplify and spread *S. entertitidis* in the layer.[59,60] According to Henzler and Opitz,[61] up to 10^5 colony-forming units of *Salmonella* may be present in a single mouse fecal pellet. Laying flocks with high levels of manure contamination are more likely to produce contaminated eggs and thus pose the greatest risk to human health.[62] Thirteen percent and 38% of *S. entertitidis* was detected on manure in layer houses in 1995 and in 1992, respectively, by the Pennsylvania Egg Quality Assurance Program; under the right conditions, this *Salmonella* sp. could be spread within the laying flock.[63]

IV. POULTRY PRODUCTION AND DISSEMINATION OF *SALMONELLA* IN THE EXTERNAL ENVIRONMENT

A. Transmission of Pathogens in Poultry Waste Streams

Since incidence of foodborne pathogens such as *Salmonella* spp. has increased over the past 20 years, it can be inferred that pathways other than those related directly to the consumption of contaminated products must be investigated.[64] Additional and unconventional pathways of increasing significance are emerging as possible sources of human salmonellosis. Land application of poultry effluent from commercial production of eggs and broilers

is the most efficient and economical way to dispose of poultry waste. There are several similarities between the concerns of pathogenicity of poultry pathogens and land application of poultry waste. The microbial flora of the poultry is being intermixed with an entirely different and diverse microflora within the soil. It is not uncommon to see pathogenic species survive for up to 12 weeks in a soil environment.[65] Bacteria from poultry can also reach groundwater and streams via this pathway. In addition, during the application of poultry effluent, there is a small risk of airborne transmission of pathogens to humans or other wildlife. Another possible pathway for the transmission of human salmonellosis is through contact with wildlife. Wild birds and rodents are often found in poultry facilities and within storage containers of feed. Wildlife also feed off of crops that are planted in soil amended with poultry litter.

B. Composition of Aerosols and Airborne Particles

Gases are widely produced directly by animals and from their feces. Ammonia, hydrogen sulfide, and methane are produced from the microbial degradation of manure.[66] Carbon dioxide is produced from both animal and microbial degradation, as well as from combustion of fuel for heating the animal house.[66] Carbon monoxide can also be produced from incomplete fuel combustion.[66] Nitrogen oxides are by-products of fuel combustion.[66] The chemical analysis of dust from pig and poultry houses is a complex mixture, which mainly yields ammonia, proteins, proteolytic enzymes, and endotoxins.[67] Endotoxins are fragments of gram-negative bacterial walls, lipopolysaccharides, which cause allergic and immunological reactions in humans.[68] Therefore, dust is a hazardous airborne agent that has allergenic agents, infectious airborne agents that has allergenic agents, infectious microorganisms, and toxic gases. The viable fraction or bioaerosol contains bacteria, fungi, and viruses.[69] Airborne microorganisms in swine houses mainly contain gram-positive bacteria with up to 40% *Streptococci* and 17% *Micrococci*.[70] Gram-negative coliform bacteria and fungi in poultry and swine house are detected in less than 1% and 13–45% of these houses, respectively.[69]

C. Airborne Transmission of Pathogenic Microorganisms

Quantitating the contribution of individual contributing factors to overall dissemination patterns is a difficult task, given the uncertainties of representative locations for measurement, fluctuations that occur over time, and unpredictable bird behavior. General aspects of pathogen dissemination in animal environments have been reviewed previously.[71] Not only should the overall airborne microbial population levels, identification of individual members,

and their pathogenic or toxin-producing potential be considered, but survival and growth activity of microorganisms during environmental stress must be considered. Although many of the microorganisms are relatively harmless to humans and birds and can be involved in ecological beneficial activities such as decomposition of waste organic matter, some of the microorganisms present can be pathogenic for either humans or birds or both. Petersen et al.[72] analyzed total aerobic microflora found in a poultry house; they detected 8% *Staphylococcus aureus* and 4 to 5% *Escherchia coli*. *Salmonella aureus* was isolated from the skin and nasal passages of poultry.[73] The transmission and survival of a typical bioaerosol organism are affected by environmental parameters that include, among other factors, air currents, temperature, and relative humidity. According to Lighthart and Mohr,[74] the more rapid the upwind speed, the more the sample concentration resembles the source concentrations. In general, airborne microorganisms can be potentially inactivated by high temperatures.[75] The response of airborne bacteria to atmospheric humidity is species dependent. For example, during the aerosolization, *Escherichia coli* K-12 was shown to survive better at low humidities than at high humidities.[76] Bacteria are more susceptible to harsh environmental stress than fungal spores and enteric viruses during transport through the air.[77]

D. Airborne Transmission of *Salmonella* spp. in Poultry Environments

Technically, airborne expression can occur within the poultry house as well from the external environment into the house and/or from the house to the external environment depending upon house structure and ventilation management. As discussed previously for the spread of *Salmonella* within the poultry-house environment, a primary mechanism of horizontal contamination of microorganism to uninfected birds is via direct contact with infected birds[24,32,33,78–81] or via contaminated drinking water.[25] However, airborne transmission cannot be ruled out as an important factor, because high bird density in a commercial poultry house may increase the disease spread by airborne microorganisms and accentuate airborne contamination.[82] Therefore, to better understand how this route influences *Salmonella* cross-contamination requires the profiling and processing of information regarding airborne bacteria and fungi typically present in the poultry house.

Airborne transmission of *Salmonella* spp. within the rearing environment may have an important role in the cross infection of birds and the maintenance of the pathogens within poultry flocks. *Salmonella enteritidis* PT4 has been shown to infect poultry[83] when carried experimentally by aerosol. Airborne contamination by *S. enteritidis* PT4 and *S. typhimurium* in general

has been experimentally demonstrated to lead to the cross-infection of birds sharing the same rearing environment.[26,84,85] The airborne coliform bacteria and *E. coli* detected in dust samples may be involved in coli-septicemia of chickens in poultry houses.[86,87] *Salmonella* detected in eggshell fragments in adjacent hatching trays was in the mixed hatching tray containing chicks from *Salmonella* inoculated eggs.[88] Laying hens exposed to aerosol containing *S. enteritidis* develop a systematic infection and excrete the organism in the fecal materials.[83] Airborne transmission of *S. enteritidis* can be spread three to four times more rapidly in molted laying hens than unmolted laying hens.[26] Mechanical fan-driven air in the hatching cabinet may move *Salmonellae* from contaminated eggs to uncontaminated eggs.[88,89] Therefore, the airborne transmission of microorganisms is regarded as a potential and favorable microenvironment route that that could lead to environmental cross-contamination and infection.[71,86,90,91]

V. FACTORS THAT INFLUENCE *SALMONELLA GROWTH AND SURVIVAL IN POULTRY ENVIRONMENTS*

A. Determinants of Microbial Competitiveness in the Poultry Environment

In poultry waste streams, sudden influxes from water effluent from lagoon spillovers or from the land application of solid wastes would suggest that a pattern of microbial blooms would parallel the associated nitrogen and phosphorus increases. Which nutrients are consistently available versus nutrients that suddenly become saturating probably dictate when opportunistic pathogens are more likely to become prominent. When fecal bacterial isolates were cocultivated with *S. typhimurium*, Ushijima and Seto[92] observed a significant decrease in *S. typhimurium* and this decreased growth was associated with competition for specific amino acids. When indigenous fecal bacteria consumed arginine, aspartate, serine, and threonine, this limited the ability of *S. typhimurium* to effectively compete. Such results would suggest that heavy influxes of organic matter could upset the balance of microorganisms in a soil ecosystem and favor opportunistic pathogens that normally would not compete as well. Nutrient availability in conjunction with a variety of environmental physicochemical factors is likely to dictate the effective concentrations of pathogens.

B. Desiccation Stress Mechanisms and Influential Factors

Desiccation is the most fundamental stressor that can be experienced by bacteria in a variety of environments. It is caused by evaporation of water from bacteria-carrying droplets, which can result in a loss of cellular

water.[93] Desiccation can stress or injur airborne microorganisms.[93–96] However, when microorganisms survive under desiccation conditions, they can be disseminated and transferred to different locations as particles become airborne.[97,98]

The rate of desiccation generally increases with increasing temperature; bacteria carrying droplets undergo desiccation regardless of environmental relative humidity (RH). However, high RH (greater than 80%) and low RH (less than 20%) are both deleterious to bacterial survival.[99] According to Benbough,[94] air stress at low humidities is attributed to the toxic effects of oxygen present, but at high RH values mechanisms lethal to airborne bacteria must be independent of the presence of oxygen. The toxic effects of oxygen may be due to free radical formation in the cell, because it has been found that metabolic inhibitors and free radical scavengers protect aerosolized bacteria.[94] There also may be irreversible changes in protein structure at high RH due to the weaker exchanges of bonded water with atmospheric water vapor.[94] More strongly held water would be involved at lower RH, so that the chances of lethal strong bond exchanges with deaths occurring are greatly increased.[99] Water loss leads to structural changes that result in membrane destabilization. Therefore, damage to surface structures can affect permeability and transport of substances into and out of a cell. Air-stressed bacteria tend to lose K^+, Na^+, and other ions by leakage through damaged membranes.[94,98] Stersky and Hedrick[96] reported that air stressed *S. newbrunswick* and *E. coli* are unable to grow on selective media containing bile salts such as sodium deoxycholate and sodium taurocholate. Water loss also causes the reduction of water activity (a_w) and thus impair cellular functions. Cell damage of air stressed bacteria can be repaired in the presence of Mg^{2+}, Fe^{3+}, and Zn^{2+}.[100] These ions help stabilize the cell envelope by providing strength and by preventing lysis.[100]

When a_w values are less than 0.84, *Salmonella* generally are unable to increase their numbers in poultry houses.[101] In addition, Hayes et al.[101] noted that a low a_w environment (less than 0.84) functions as a physical barrier to the establishment or continuation of *Salmonella* contamination in a poultry house. In contrast, a high a_w environment may provide a high risk of continuing *Salmonella* contamination of poultry, provided the organism is introduced into the favorable environmental condition in a poultry house.[101] Desiccation can reduce the number of *Salmonella* in poultry associated environments such as manure and litter.[102] However, *Salmonella* spp. may be able to survive and adapt to more severe desiccation conditions. Juven et al.[103] reported that the survival of *Salmonella* was greater at a_w of 0.43 than at 0.75. *Salmonella* spp. may survive for greater than 120 days in spray-dried milk and greater than 6 months in chalk.[104,105] When surviving in a desiccated state, *Salmonella* spp. could be transmitted or could infect new flocks via dust, feed, litter, feather, or dust particles.

VI. DETECTION AND SAMPLING METHODS OF *SALMONELLA IN POULTRY ENVIRONMENTS*

A. Culture-Based Identification of *Salmonella*—Special Considerations for Samples Originating From Poultry Waste Environments

In general, there are two approaches to analyze the effluent samples collected for analysis of pathogen impact, namely, presence of fecal organisms as indicators of animal waste contamination, and testing of effluent for effects on pathogen sustainability with specific marker strains for representative pathogens. Presence of fecal indicator organisms has been described by Tiquia et al.[106] Briefly, after diluting water samples,the resulting dilutions are plated on the appropriate selective media for fecal indicator organisms. *Escherichia coli* coliforms can be assayed by direct plating on selective 3M petrifilm (3M Corporation) and fecal streptococci can be plated on m-Enterococcus agar (Difco, Detroit, MI). Log_{10} values of colony-forming units are usually determined after incubation for at least 48 h at 37°C.

To achieve effective recovery and more specific quantization of *Salmonella* spp. from waste environments requires several considerations. First of all, the choice of media will depend on the levels and types of background organisms and the ability to create selective conditions that discourage growth of these organisms while allowing the growth of the organism of interest, in this case *Salmonella* spp. However, the selective media conditions must not be so severe as to prevent recovery of injured bacterial cells, a real possibility with waste environments that would generally be considered a poor growth environment. Consequently, isolation and identification procedures for most organisms such as *Salmonella* spp. usually have an initial enrichment step that consists of medium ingredients that will enhance recovery by supporting revival and optimal growth of stressed and injured bacterial cells followed by transfer to a selective medium that only supports growth of the organism of interest.

When attempting to recover *Salmonella* spp. and other bacteria from soils and similar environments, a primary concern is interference or overgrowth by indigenous fungi that would be found particularly in aerobic waste environments such as the surface of composting poultry litter or after soil application of poultry manure or slurry waste. Most commercial selective media developed for salmonellae have been developed for recovery from food or clinical samples, which would be much less likely to contain substantial numbers of fungi. Consequently, these media can be easily overgrown by fungi when used for environmental samples. This problem can be resolved by incorporating an antifungal agent into the commercial media. The key is that the antifungal agent has a fairly broad spectrum against most indigenous environmental fungi but does not inhibit growth of the bacteria

being recovered. The antifungal agent cycloheximide, which has been used in soil microbial analysis to inhibit fungi,[107] was tested in a series of studies as a potential fungal inhibitor for differential plating of samples from animal feeds.[108,109] Ha et al.[109] observed that cycloheximide completely inhibited indigenous fungal overgrowth on aerobic bacterial plates from a variety of feeds. Growth rates of *S. typhimurium* in minimal mineral media were significantly decreased by addition of cycloheximide aerobically and anaerobically, but *Salmonella* recoveries on brilliant green agar, MacConkey agar, selenite cysteine broth, and tetrathionate broth were not affected by cycloheximide additions at concentrations up to 1000 mg/L.[108]

B. Molecular Methods

Molecular methods entail the extraction and use of the genetic material of the organism. The ability of a DNA probe of known sequence to hybridize with a strand on genomic DNA from an unknown organism is evaluated where the probe is labeled either by radioisotope or with a reporter group such as alkaline phosphatase, peroxidase, biotin, and other compounds capable of yielding a visible response when hybridization has occurred.[110,111] Hybridization is the basis for some of the commercial kits used in clinical microbiology, and colorimetric and radiolabeled probes have been successfully used to identify foodborne salmonellae serovars.[111–113] A specific 16sRNA sequence biotin labeled DNA probe was successfully used to identify salmonellae recovered from thermophilic composts by detection of hybridization in a biotin colorimetric assay.[114] A number of nucleic acid probes to identify a majority of foodborne bacterial pathogens are available, but current hybridization techniques require at least 10^3 to 10^4 copies of the target sequence to yield an accurate result.[115–117] Consequently, some culture preenrichment is required to achieve bacterial cell numbers high enough to provide sufficient DNA copies.[115–117]

To achieve much lower detection limits requires amplification of the target DNA to a quantity detectable by conventional techniques. Polymerase chain reactions (PCRs) are basically the in vitro amplification of target chromosomal DNA via thermostable DNA polymerase in combination with specific oligonucleotide primers. The significant advantage of gene amplification over hybridization gene probes is its potential of detecting fewer specific organisms even in the presence of large numbers of nontarget organisms.[117] Among the molecular methods that are currently available, polymerase chain reaction or PCR represents a tremendous potential for the detection of low levels of pathogenic bacteria within complex environmental backgrounds.[117,118] PCR has had only limited application directly with animal waste environment microbial detection, but Ng et al.[119] used PCR to amplify 16S rRNA sequences to identify bacteria from sewage-sludge anaerobic

digester contents. However, PCR technology has been used for detection of microbial pathogens in a variety of matrices, including soils, water, foods, feeds, and clinical samples.[117,118,120–124] Consequently, numerous protocols and modifications of protocols to optimize sample analysis have been reported over the years. Most changes and alterations for improvement have focused on temperature cycling profiles, DNA polymerases, oligonucleotide primers and sequences, and sample extraction and processing.[117,120,125,126]

Most of the efforts associated with PCR detection of *Salmonella* spp. have been directed toward analysis of food and clinical samples. Two issues that have been encountered in these types of samples have the potential to plague poultry environmental analyses as well. The first issue concerns the specificity of the primer for the target organism. Ideally, primer sequence should conserved sufficiently to include most strain variances possible for the organism of interest but should retain enough specificity to not react with organisms not identified as the target organism. Oligonucleotide primers constructed from the *hns* gene of *Salmonella* spp.[127] have been shown to specifically amplify *Salmonella* spp. DNA by PCR.[120] However, Maciorowski et al.[123] found that these primers detect not only *Salmonella* spp., but also *Clostridium* spp., and Endley et al.[128] found *fimA* primers to be more suitable for specific detection of *Salmonella* in animal waste environments. Since *Clostridia* spp. have been shown to serve as an excellent fecal pollution microbial indicator,[129] *hns* primers have been proposed to screen samples for the presence of *Salmonella* spp. as well as microbial pollution from animal feces.

A second issue is interference of amplification by the nature of the sample. Generally, since gene amplification-based screening procedures from environmental and clinical samples are vulnerable to interference by organic matrices.[22,117,123,130–133] Essentially, interference implies that inhibitory compounds are either directly bound to the target nucleic acids or are indirectly inhibitory to the polymerase enzyme. Approaches to alleviate this problem have been described in detail by Pillai and Ricke[117] and are only discussed briefly here. Traditionally, elimination of sample interference requires direct extraction of the nucleic acids by the use of extended purification steps with combinations of organic reagents such as chloroform and phenol. Given the time required to do these steps, a second approach has led to protocols where a rapid separation of bacterial cells from the sample matrix is done initially followed by a much more rapid bacterial cell lysis step. In addition, culture preenrichment is often included to increase the concentration of target DNA and dilute the sample matrix background prior to conducting PCR.

Current technological developments may further resolve interference problems. Techniques involving more sensitive detection methods are more specific than the visual detection of stained bands on electrophoretic gels. Such sensitivity may be required to confirm the presence of low-level DNA

amplification in matrices that permit only a low amplification efficiency. One system currently under development provides for the solution hybridization of two probes, one labeled with biotin at the 5′ end and the other labeled with fluorescein at the 5′ end.[123,134–139] The hybridized DNA is then bound by its biotin label to a biotinylated membrane stick and the fluorescein label is allowed to bind to a polyclonal anti-fluorescein antibody conjugated to urease.[136] The presence or absence of the amplicon specific for the *hns* sequence is based on the incremental changes in pH (measured in microvolts per second) resulting when the urease-containing sandwich is exposed to urea in a pH-sensitive potentiometric sensor.

The rapid detection of microbial pathogens in complex environmental matrices by gene amplification protocols is presently far from optimal due to interference from organic matrices. Present detection procedures require between 16 and 20 h for preenrichment and between 24 and 48 h for selective enrichment for *Salmonella* spp.[140–142] PCR amplification and oligonucleotide probes have successfully detected both artificially seeded and naturally occurring *Salmonella* spp. within oyster meat without preenrichment[120] or in poultry meat using pretreatments and only 4 h of preenrichment.[143] Once optimized, the combination of PCR and a dual hybridization probe assay may be used to rapidly, reliably, and inexpensively screen a variety of poultry waste streams for the presence of *Salmonella* spp.

C. Conventional Sampling Methods for *Salmonella* Detection in Poultry Production Environments

Effective detection of *Salmonella* in a poultry environment is highly dependent on representative sampling that achieves true assessment of actual contamination levels. Conventional techniques of direct culturing of litter and drag-swab sampling are mainly used to evaluate the contamination of poultry houses, and to detect *Salmonella*. *Salmonella infantis*, *S. bredeney*, *S. havana*, *S. Johannesburg*, *S. montevideo*, and *S. drypool* were isolated in the culture of 6-week-old floor litter at the poultry farm.[144] In broiler flocks, *S. typhimurium* and *S. havana* contamination was detected in 9 out of 13 sheds by drag swab and in 7 out of 13 sheds by litter culture.[145] Caldwell et al.[146] found that *Salmonella* was more likely to be detected by drag swab testing in occupied poultry houses than in the vacant poultry houses. The presence of chickens may play a major role in the amplification and dissemination of *Salmonella* to a greater area of the poultry house.[146] Additionally, feed contamination or early neonatal infection of chicks at the hatchery may also be important.[146] Hayes et al.[101] compared drag-swab and litter sampling methods for detection of *Salmonella* spp. in commercial poultry houses. Drag-swab testing detected *Salmonella* spp. more often than did litter culture. The contamination with *Salmonella* spp. from the farms sampled

was detected in over half the houses when combining the results of the two testing methods.[101]

D. Bioaerosol Sampling

Conventional environmental sampling techniques present difficulties in ease of sampling and sampling size due to the physical limits of the site to be collected from.[145,146] Efficient collection of microorganisms from the air to monitor airborne microorganisms offers an alternative approach that takes into account overall contamination levels. An appropriate method for analysis of air samples is also necessary. However, the variety and complexity of bioaerosols complicate monitoring and exposure assessment.[147] The types of air sampler used for collection may also be critical, and the factors that must be considered have been reviewed extensively by Pillai and Ricke[71]. According to Woodward et al.,[148] overall aerosol bacterial counts were higher when collected by impactors before adjusting for airflow rate and higher yields of total bacterial colony-forming units were recovered from impingers after being recalculated for airflow rate. However, Woodward et al.[148]noted that the further development of aerosol sampling systems may allow for continuous monitoring of microbial populations in a poultry layer house.

When airborne microorganisms are conventionally detected by total count and culture techniques, the microorganisms that are not culturable under the specific growth conditions used in the laboratory remain undetected.[149] The microorganisms may be exposed to the stress of aerosolization and sampling, which may result in a loss of culturability.[150,151] In addition, culture-based techniques can take several days to weeks to detect and identify a specific airborne microorganism. Therefore, PCR amplification represents specificity, sensitivity, and reduced processing time for aerobiological monitoring of small numbers of targeted microorganisms.[147] Detection by PCR methods is very sensitive to interference by the complex organic load in either litter or drag-swab samples.[22,117,123,132,138,152] Therefore, airborne sampling has been proposed as an alternative to solve the sample size labor and cost.[133] Because this technique is an environmental sampling method, airborne sampling potentially provides representative monitoring of either the interior poultry house or the environmental boundary areas surrounding the poultry facilities. Cason et al.[88] and Berrang et al.[89] reported that *Salmonella* was transmitted from contaminated eggs to uncontaminated eggs during hatching and this transmission may be through fan-driven air. Airborne sampling techniques have exhibited less interference when combined to PCR amplification.[153] Kwon et al.[132] reported that when PCR was conducted by spiking samples of *S. typhimurium*, PCR amplification of *Salmonella*-specific DNA was more readily detected in air filter samples than in litter samples.

VII. TRACKING *SALMONELLA IN THE POULTRY ENVIRONMENT*

A. Generation of Antibiotic Resistant Marker Strains

Developing an antibiotic-resistant bacterial strain of the organism of interest is a useful tool for constructing "marker" strains that can easily be followed in a laboratory or during in situ studies for following survival as well as genetic stabilities of organisms under environmental stresses.[154–161] Antibiotic resistant marker strains can be generated in any number of ways including selection for naturally resistant or selection for spontaneous mutants in the presence of the antibiotic.[162] These mutants generally contain a functional gene that is mutated in a way that confers resistance to a specific strain.[162] Examples include chloramphenicol point mutations in 23S rRNA genes that remain functional and rifampicin- or nalidixic acid-resistant bacteria with mutations in RNA polymerase or DNA gyrase genes.[162]

More directed means of introducing antibiotic resistance include insertion of plasmids containing respective genes that encode for specific and selectable genes conferring antibiotic genes. Such approaches have allowed for the selective growth, recovery, and quantitation of *E. coli* lysine auxotrophs in the presence of poultry feed backgrounds.[163,164] However, without maintenance of specific antibiotic-selective conditions, plasmids can be lost as bacterial cells undergo cell division. Insertion of antibiotic resistance genes into the chromosome can be accomplished by transposition with mobile transposons with selectable antibiotic genes.[165,166] In addition, transposons, depending upon where they become inserted in the chromosomal genome, can result in the loss of phenotype by insertion mutagenesis into functional genes and essentially "knocking" them out. With such an approach, selective screening has allowed the discovery of numerous genes and new functions of known genes,[167] and, depending on the nature of the genetic construct, not only can a marker organism be followed but expression of individual genes can be followed in the environment. Further refinement involves replacing the antibiotic resistance marker with a measurable compound such as products produced by an inserted enzyme or luminescence from inserted *lux* genes. The specific aspects of this approach are discussed in more detail in the section on genetic methods.

B. Application of *Salmonella* Antibiotic Strains in Poultry Environmental Studies

Antibiotic marker strains of *Salmonella* poultry isolates have been used for survivability studies in poultry feed matrices, chick cecal colonization, and soil microcosm studies.[108,109,168–175] The strain used in most of these studies was a primary poultry isolate of *Salmonella typhimurium* that was originally isolated for resistance to novobiocin and nalidixic acid.[176] For poultry

infection studies this organism is typically introduced to the birds orally or through environmental contact, and after several days are allowed for colonization and establishment; birds are killed and intestinal contents and organs are examined for the presence of the marker strain. Recovery on *Salmonella*-selective media containing the two antibiotics expedites rapid enumeration of the marker strain of *Salmonella*. For survivability studies the marker strain can be prepared as a liquid or dry inoculum and inoculated into the respective nondiluted environmental samples and survivors enumerated.[108,170,174,175] This allows inoculation and incubation of environmental samples directly without having to sterilize the sample first. Such an approach allows for the direct enumeration of *Salmonella* without background bacterial populations coming up on the plates.

VIII. STRATEGIES FOR STUDYING SURVIVABILITY AND GROWTH OF *SALMONELLA IN THE POULTRY ENVIRONMENT*

A. Kinetics of Survivability

Once a marker *Salmonella* strain has been constructed, it becomes possible to assess and quantitate of the target organism not only in the laboratory but in the environment potentially as well. For laboratory studies to determine survivor kinetics, liquid microcosm studies designed to simulate poultry environments can allow for growth to be measured turbidimetrically on a spectrophotometer in aerobic or anaerobic batch culture and growth rate estimated by linear regression analysis.[177,178] To estimate survivability after logarithmic growth (defined as stationary phase where net multiplication of bacterial cells no longer occurs), total cell counts can be determined as a direct microscopic count using a calibrated slide counting chamber on diluted samples. Viable cell counts can be determined by serial 10-fold dilutions in the appropriate physiological buffer and plating the various dilutions on recovery medium. Although dependent on the type of organism for a typical heterotroph found in the gastrointestinal tract, viable total cells are usually enumerated for a period of at least 5 days after stationary phase from liquid cultures. From these data a survivability time as a constant can be estimated (ST_{50}), which is defined as the time for 50% of the initial viable population to become nonviable and can be calculated from the fractional turnover (m) of the regression line of viable cell number where $ST_{50} = \ln 2/m$.[179,180] Cell numbers and optical densities expressed as logarithmic functions can also be subjected to linear regression statistical (least squares) analysis to assess the lack of fit of the regression line.[108,177,178]

B. Bacterial Culture Techniques to Simulate Waste Environments

Understanding pathogen responses in growth-limiting conditions that are usually prevalent in environments associated with poultry waste streams

requires culture systems that will allow for extended periods of slow growth. A culture system that is specifically designed to solve this problem is continuous culture. Continuous cultures represent growth culture systems where substrate availability is held constant by mechanical control of inflow and outflow. This results in bacterial cell number per unit substrate in the growth vessel being maintained at steady-state growth conditions. Growth medium is formulated such that one nutrient is set to be the first growth-limiting nutrient. The essentiality of continuous culture operation is that at steady state the nutrient concentration is low enough that each drop of fresh medium into the growth vessel is quantitatively only enough nutrient to be instantaneously consumed by the bacterial population without an increase in cell number, hence the "steady-state" nature of the growth vessel bacterial population. This results in growth of the bacterial population that is proportional to dilution rate and therefore can be controlled by the infusion rate of the pump. Consequently, growth rate can be controlled and other factors that may influence bacterial growth physiology can be examined independently.

Most of the early applications of continuous culture systems involved studies to understand fundamental physiological questions on single pure cultures of bacteria.[181–184] However, various continuous culture systems and approaches have become widely used for cultivating microbial communities from a variety of environments to study complex mixtures of microbial consortia.[183,185–195] Interactions between pathogens and indigenous microbial consortia have generally been limited to interactions among intestinal bacteria and specific pathogens,[92,196,197] cultivation of pure cultures of pathogens under anaerobic gastrointestinal incubation conditions,[198] or development and study of probiotic cecal cultures antagonistic to colonization of *Salmonella* spp. in the ceca of the young chick.[168,199–201]

Applications more relevant to animal waste environments have been almost exclusively focused on the development of semi-solid systems that allow the flow of fluid through a reactor vessel designed to retain bacterial aggregates and anaerobic digester granules.[195,202] The goal of many of these studies has been to examine the efficiency of methane production as a function of the environmental restraints unique to the slow entry and turnover of solids, ambient temperatures, high concentrations of potentially toxic fermentation intermediates, and the use of attached-film to retain slow growing bacterial populations.[203–211] To address the issue of proliferation and survival of pathogens in waste streams will require combining the continuous culture methodology developed for slow growing microbial consortia and adverse environmental conditions characteristic of waste steam environments with the use of marker strains for pathogens of particular interest. This will allow for the ability to predict the sustainability of pathogens being emitted by confined food animal operations, as well as the response of pathogens already present in the environment after exposure to the waste stream effluents.

IX. UNDERSTANDING *SALMONELLA PATHOGENESIS IN THE POULTRY ENVIRONMENT*

A. Virulence and Pathogenesis Defined

Virulence is essentially the ability of a pathogen to become invasive (in the case of foodborne pathogens actually penetrate epithelial cells that line the gut) and elicit harmful effects to the host because of that invasion. Virulence is a complex phenotype generally made up of a combination of bacterial cellular components that make it possible for the bacterial cell to come in contact with and adhere to the target host cell (motility and attachment proteins), invade the host cell (production of invasion proteins), and cause harm or outright kill the host cell (production of toxins).[212]

In general, environmental stresses and starvation conditions can induce virulence genes in most of the pathogens that have been studied, but there have only been minimal direct studies on virulence expression in animal waste environments. For example, isolation and survival studies indicate that the highly virulent *Escherichia coli* O157:H7 can be disseminated in cattle manure and manure slurries. In addition, *E. coli* O157:H7 strains have been shown to survive in manure for several months and retain their ability to produce toxins.[213,214] Such observations are compounded by evidence that this pathogen may also have unusual tolerance against some environmental stresses such as acidic and dry conditions.[213,215,216] Consequently, when pastures are irrigated with cow manure slurries or vegetable gardens are fertilized with cattle manure, soil environments may be created that perpetuate the more virulent forms of *E. coli* for potential infection of humans.[213,214,217,218]

Salmonella spp. do have the ability to become more virulent under certain environmental conditions that could conceivably occur in animal waste environments, such as low oxygen, high osmolarity, and slightly alkaline pH,[219–222] and high concentrations of short-chain fatty acids (similar to concentrations encountered in the intestinal tract and colon) will influence attachment and invasion of epithelial cells in tissue cultures.[223–225] It would not be surprising if environmental conditions encountered by *Salmonella* spp. after excretion from the bird and during waste processing would either induce virulence in these *Salmonella* strains and/or favor selection toward subpopulations of more virulent strains. The following sections address the methodology available for studying virulence expression under the environmental conditions encountered in poultry waste management.

B. Application of Virulence Fusion Assays in Poultry Studies

Salmonella pathogenesis genes have been used in genetic fusion assays for monitoring expression of virulence. Expression of served virulence genes

is regulated by HilA, a transcriptional activator encoded within SPI1.[220,222] InvF is known to be required for the invasion of epithelial cells.[220,222,226–228] Durant et al.[228] examined the effects of short-chain fatty acids (SCFA) on the expression of *hilA* and *invF-lacZY* transcriptional fusions, to determine the potential role of SCFA in the pathogenesis of *S. typhimurium*. At pH 7, *hilA* and *invF* expression was induced by acetate but not by propionate or butyrate, while at pH 6, all SCFA induced *hilA* and *invF* expression. The pH-dependent manner of induction suggests that entry of SCFA into the cell was necessary for induction. Consequently, it is possible that SCFA may serve as an environmental signal that triggers the expression of invasion genes in the gastrointestinal tract. This would also suggest that waste stream environments where fermentation might occur, such as the environments characteristic of deeply stacked litter, deep sediments in lagoons, or anaerobic digesters, could be conducive to enhanced virulence.

A series of studies has also been conducted on determining HilA expression in poultry in vivo intestinal studies and in vitro laboratory simulations of chicken intestinal conditions using a poultry isolate of *S. enteritidis* carrying a *hilA-lacZY* transcriptional fusion from *S. typhimurium*.[229–231] Durant et al.[229] observed that feed withdrawal alters the environment of the crop by causing significant reductions in the *Lactobacillus* population along with decreased lactate concentrations and increased pH. These changes in the crop of molted birds were accompanied by significant increases in *S. enteritidis* colonization of the crop (sixfold increase in the number of *S. enteritidis* positive hen crops) and ceca (3 $\log_{10}$ increase in *S. enteritidis* colonization), along with increased invasion of the spleen and liver. In addition, expression of *hilA* was nearly doubled when *S. enteritidis* was grown in filter-sterile crop contents of the molted birds compared to unmolted birds.[229] When the effects of pH, carbohydrate sources, amino acids, and lactate on *hilA* expression of *S. enteritidis* virulence were examined in the laboratory, addition of 0.2% glucose, fructose, or mannose reduced *hilA* expression 1.5- to 2-fold.[230] Lactate reduced *hilA* expression at pH 6, 5, and 4, with the lowest expression occurring at pH 4. Such results suggest that the composition of the crop lumen, which is determined by the food ingested, may be important for determining the potential for subsequent *S. enteritidis* infection. The results also suggest that the expression of *S. enteritidis* virulence genes does not depend on a single environmental stimulus in the crop, but that there may be interactions among the stimuli. Based on these results it appears that the arrival of *S. enteritidis* into a crop with a low pH, as a result of high concentrations of lactate, could reduce virulence gene expression. This has led to development of several molting dietary regimes that still induce molt in the laying hen but support a fermentative gastrointestinal environment in the laying hen that can serve as a barrier to *Salmonella* colonization.[232–242]

C. Genetic Approaches to Studying Environmental Survival and Pathogenesis

Detection of *Salmonella* gene expression will become a more useful concept as more research is done. It is possible that the expression of these genes elicits an enhanced pathogenic response from *Salmonella*, which can in turn cause concern in public health and pathogen control. The knowledge of genetic virulence expression will also be of great importance for assessment of food safety risk to determine whether application of poultry wastes to the environment plays a role in the triggering of the *Salmonella* spp. virulence that has potential of transmission to poultry meat and egg production.

Transposon footprinting method is a novel and efficient genetic approach for identification of bacterial genes which are required for survival in diverse harsh conditions.[243] This transposon footprinting method could be applied to a stressful nongrowth environmental condition typically encountered by *Salmonella* spp.[244] Kwon and Ricke[166] devised an efficient PCR-based method for specific amplification of transposon-flanking sequences. This method requires the sequence information of only transposon-specific sequences. It consists of two simple steps of ligation and amplification and does not exhibit nonspecific background amplification. It can amplify multiple independent insertions either within a mutant or in a pool of multiple mutants. This method, termed transposon footprinting, simultaneously amplifies the transposon-flanking sequences in a complex pool of the transposon mutants. Because the length of the amplified DNA fragment is unique for each distinct transposon mutant, the PCR products can be separated on an agarose gel to generate a transposon footprint, with each band in the footprint representing the corresponding transposon mutant. The missing DNA band(s) in this footprints can easily be visualized on the agarose gel and the respective mutants identified.

Transposons are defined as DNA elements that can move or transpose from one place in DNA to a different place with the action of transposase enzymes.[245] Because they also have an insertion element at each end, transposons can readily move from place to place carrying their genes with them. The transposon itself usually encodes its own transposases, so that it carries with it the ability to move each time it moves. For this reason, transposons have been called "jumping genes."[246] In all transposition events, the transposase enzyme cuts the donor DNA at the ends of the transposon and then inserts the transposon into the target DNA. When transposons are experimentally inserted into the genome of bacteria, target genes result in a loss of function, and downstream genes, sequences that lie in the 5′ direction on the coding strand of a DNA region, in an operon, a region on DNA encompassing genes that are transcribed from the same promotor, could be affected by the inserted transposon.[247] An altered phenotype mutant resulting from the constitutive expression, a gene expressed constantly,

of downstream gene or genes can be isolated if the transposon has a constitutive promoter, a region on DNA to which RNA polymerase binds to initiate transcription.[220] For the identification of the sequence that flanks the transposon, genes affected by the inserted transposon should be characterized when a transposon mutant with the altered phenotype of interest is isolated.

Now that more detailed information on the *Salmonella* genome is known including the complete sequence of some strains[248] application of genetic methodology for environmental characterization of *Salmonella* spp. will not only yield more in-depth information on the presence of *Salmonella* spp. but also allow quantitation of specific genes using microarray technology.[249–250] Use of PCR methods to quantitate *Salmonella* gene expression in backgrounds such as poultry feeds, as well more sophisticated immunological and cultural techniques, will allow for more extensive monitoring of *Salmonella* spp. during the poultry production cycle.[220,251–252]

CONCLUSIONS

Based on what has been discussed in this review, the following recommendations can be emphasized for further studies in *Salmonella* spp. in poultry environments. To address the issue of proliferation and survival of pathogens in environments will require combining the continuous culture methodology developed to simulate slow-growing microbial consortia and adverse environmental conditions characteristic of poultry production environments actually encountered using marker strains for *Salmonella* spp. of particular interest. In addition, the biology of *Salmonella* spp. needs to be examined in the context of the environments that would be expected to be found in the poultry waste holding facilities and waste streams. Currently, knowledge of *Salmonella* metabolism, physiology, and genetics is largely based on studies conducted under aerobic, nutrient rich, laboratory medium conditions. Basic information is lacking on metabolism, physiology, and genetics while growing in environmental conditions consistent with poultry production and waste streams. However, a wealth of molecular information and techniques is available to dissect key results from microcosm and chemostat culture studies that approximate environmental conditions consistent with the characteristics associated with lagoons and waste streams. There already is a considerable amount known about environmental signals that impact starvation and survivability, as well as the signals that control growth during and after invasion of human intestinal cells.[212,253–257] However, any attempt to develop accurate predictive models will require generation of extensive in vitro and in vivo data on the biology of *Salmonella* spp. under poultry environmental conditions.

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